Androgen deprivation therapy has no effect on Pim-1 expression in a mouse model of prostate cancer

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Abstract. The aim of the present study was to observe the dynamic changes of proto-oncogene, serine/threonine kinase, Pim-1 at the gene and protein level in a mouse model of prostate cancer following surgical castration. Using LNCaP cells to establish a subcutaneous xenograft model and orthotopic prostate cancer BALB/c nude mouse models, the xenograft models were divided into an androgen-dependent prostate cancer group (ADPC), an androgen deprivation therapy (ADT) group and an androgen independent prostate cancer (AIPC) group. Reverse transcription-polymerase chain reaction (RT-PCR), RT-quantitative PCR, ELISA and immunohistochemistry analyses were performed to compare the expression levels of Pim-1, prostate-specific antigen (PSA) and androgen receptor (AR) in tumor tissue of three subgroups. Agarose gel electrophoresis revealed that the RT-PCR results of the ADPC (0.59±0.01) and AIPC groups (1.14±0.015) were significantly different when compared with the ADT group (0.62±0.026; P<0.05). As for RT-qPCR, the ΔCq of Pim-1 in the ADPC (6.15±0.34) and AIPC (4.56±0.23) groups were significantly different compared with the ADT group (5.11±0.21; P<0.05). Using $2^{-\Delta\Delta Cq}$ as a relative quantification method to analyze the data, the amplification products of Pim-1 increased by 2.05 and 3.01 times in the ADT and AIPC groups, respectively. ELISA demonstrated the following: The serum concentration of PSA was 0 ng/ml in the control group, 0.48±0.025 ng/ml in the ADPC group and 0.87±0.023 ng/ml in the AIPC group, which were significantly different compared with the ADT group (0.17±0.032 ng/ml; P<0.01). Upon immunohistochemical staining, the protein expression levels of Pim-1 and AR, respectively, were 0.017±0.0021 and 0.032±0.009 in the ADPC group, 0.024±0.0019 and 0.040±0.011 in the AIPC group, and 0.018±0.0013 and 0.019±0.006 in the ADT group. The protein levels of Pim-1 and AR in the ADPC and AIPC groups were significantly different compared with the ADT group (P<0.01). In addition, an orthotopic prostate cancer animal model of ADT was successfully established in the current study, and further investigation revealed that ADT did not affect the expression of Pim-1 at the gene or protein levels; thus, it is hypothesized that Pim-1 may be important in the proliferation and differentiation of prostate cancer during ADT.

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Introduction

Human proviral integration site for Moloney murine leukemia virus (Pim) is a proto-oncogene that was initially recognized 20 years ago (1); further studies have revealed that *Pim* encodes serine/threonine kinase and functions as a signaling regulator through cellular substrate phosphorylation (2,3). Pim participates in multiple biological functions, including numerous cellular signaling pathways, and regulates cell cycle progression, cell proliferation and differentiation, and inhibits apoptosis. Recently, the association between androgen receptor (AR) and the proto-oncogene, serine/threonine kinase, *Pim-1*, has been shown to be strong, and studies have indicated that Pim-1 is expressed in androgen-dependent prostate cancer (ADPC) and castration-resistant prostate cancer (CRPC) (4). However, the response of Pim-1 to androgen deprivation therapy (ADT), particularly the dynamic changes in animal models, has not previously been established. In the current study, reverse transcription-quantitative polymerase chain reaction (RT-qPCR), enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry (IHC) were performed to detect the difference in expression levels of Pim-1 in mice models, in which prostate cancer was simulated to develop into CRPC during ADT. In addition, the present study discusses the known molecular mechanisms of Pim-1 kinase in prostate tumorigenesis and progression, providing the opportunities for targeting *Pim-1* as an alternative therapeutic method for prostate cancer, particularly in CRPC cases.

Materials and methods

Lab preparations. A total of 32 male BALB/c nude mice were purchased from the Resources Research and Development Center of the Institute of Laboratory Animal, Chinese Academy of Medical Sciences (Beijing HFK Bioscience Co., Ltd., Beijing China; animal certification no. SCXK2009-0004). All procedures involving mice were approved by the University Committee on Use and Care of Animals at the Tianjin Medical University, China, and conform to all regulatory standards. LNCaP cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Chloroform, isopropanol, RNase-free ddH_2O and TRIzol were purchased from Tiangen Biotech Co., Ltd. (Beijing, China). cDNA synthesis and the qPCR kit were purchased from Fermentas (Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). The following primers were purchased from Shanghai Sangon Biotech, Co., Ltd. (Shanghai, China): Upstream, 5'-GCCTCAACTCCTCCCATAGATAC-3' and downstream, 5'-GCGGCATTCAGCAGAACTCAT-3' for Pim-1 (product length, 147 bp); upstream, 5'-TGACGTGGACATCCGCAAAG-3' and downstream, 5'-CTGGAAGGTGGACAGCGAGG-3' for β-actin (product length, 205 bp).

Animals. Thirty-two male BALB/c nude mice (age, 6 weeks; weight, 16-18 g) were randomly divided into four groups: ADPC, ADT, androgen-independent prostate cancer (AIPC) and control groups, with 8 mice per group. Mice were housed at 25˚C under a 12-h light/dark cycle and were fed and watered on schedule.

Xenograft tumor model in nude mice. LNCaP cells ($1x10^6$ cells/mouse) were suspended in 0.1 ml serum-free RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) and implanted subcutaneously into the flank of each mouse. The mice were maintained in ventilated cages and fed normal food and water for ~4 weeks after tumor inoculation to obtain a subcutaneous tumor. The tumor volume was determined by caliper measurements and divided into three equivalent segments to implant into the anterior capsule of the nude mice prostate in the ADPC, AIPC and ADT groups. Meanwhile, the tumor tissue was implanted in subcutaneous tissue to assess growth changes of orthotopic prostate cancer indirectly. In the ADPC group, the prostate tumor was removed 8 weeks after implantation. In the ADT group, the mice were castrated by surgery 8 weeks after implantation and the prostate tumor was removed 3 days later, following castration. In the AIPC group, the mice were castrated 15 days after tumor implantation and subsequently the prostate tumor was removed 8 weeks later (Fig. 1). The tumor volume was calculated based on weekly caliper measurement using the following formula: Volume = ($width^2$ x length) / 2. All tumors from the three individual groups were assessed by pathologists at the Tianjin Institute of Urology, China. RT-PCR was conducted to evaluate the mRNA expression levels of Pim-1 and IHC was performed to analyze its protein expression.

RT-qPCR. Fresh prostate tumor tissue samples were immediately immersed into the RNA later solution (Qiagen, Inc., Valencia, CA, USA) for 24 h at 4˚C and total mRNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. A Multisource Total RNA Miniprep kit (Axygen; Corning Life Sciences, Shanghai, China) was used in the AIPC, ADPC and ADT subgroups. The concentration of RNA was measured using an ultraviolet spectrophotometer (Biophotometer; Eppendorf, Hamburg, Germany). cDNA was synthesized using the High-Capacity cDNA Synthesis kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR was performed using a one-step RT-PCR system (Beijing Transgen Biotech Co., Ltd., Beijing, China). PCR was run in a 12.5 μl reaction volume. The PCR specific primer pairs (Takara Biotechnology Co., Ltd., Dalian, China) were 5'-GCCTCAACTCCTCCCATAGATAC-3' (forward) and 5'-GCGGCATTCAGCAGAACTCAT-3' (reverse). The PCR conditions were as follows: Preprocessing of uracil-DNA glycosylase at 50˚C for 2 min; pre-denaturation at 95˚C for 10 min; 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec; and full extension at 72˚C for 10 min to complete the amplification.

Analysis of RT-qPCR. The PCR products were separated on 1% agarose gels containing ethidium bromide using the TC-96/G/H(b)A electrophoresis apparatus (Beijing Liuyi Biotechnology Co., Ltd., Beijing, China). The gels were photographed and analyzed using a Tanon-1600 gel imaging analysis system (Shanghai Tanon Science & Technology Co., Ltd., Shanghai, China) by measurement of the gray value of the electrophoresis strip. The ratio between the target gene and β-actin was used to calculate the relative quantitation. The amplification products and the relative expression of RT-PCR were analyzed using the ΔΔCq method (5).

ELISA of mice plasma prostate-specific antigen (PSA). Mouse plasma was obtained from the caudal vein every 2 weeks for measurement of PSA levels using a Quantikine human PSA immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. Concentration of Pim-1 protein was measured using a commercially quantitative ELISA kit (BioCheck USA, Scarborough, ME, USA), according to the manufacturer's instruction. A total of three independent experiments, each in triplicate, were assayed and the median Pim-1 protein concentration from each duplicate was used for statistical analysis.

IHC. Tumor tissue was embedded in paraffin and sliced into 3-μm thick sections for IHC. Sections were dewaxed in xylene, hydrated through graded alcohols and rinsed in deionized water. Antigen retrieval was performed by boiling the slides in a cooker for 10 min in a citrate buffer (pH 6.0; Wuhan Boster Biological Engineering Co., Ltd., Wuhan, China). Subsequent to a 10-min treatment with 3% H_2O_2, tissue sections were blocked with 5% normal goat serum (Genomapping Technology Co., Ltd. Tianjin, China) in Tris-buffered saline (pH 8.0; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Tianjin, China) for 1 h at room temperature, incubated with rabbit polyclonal antibody AR (dilution, 1:100; cat. no. ab133273; Abcam, Cambridge, MA, USA) and rabbit monoclonal antibody Pim-1 (dilution, 1:2,000; cat. no. ab75776; Abcam) at 4˚C overnight, and incubated with horseradish peroxidase-conjugated

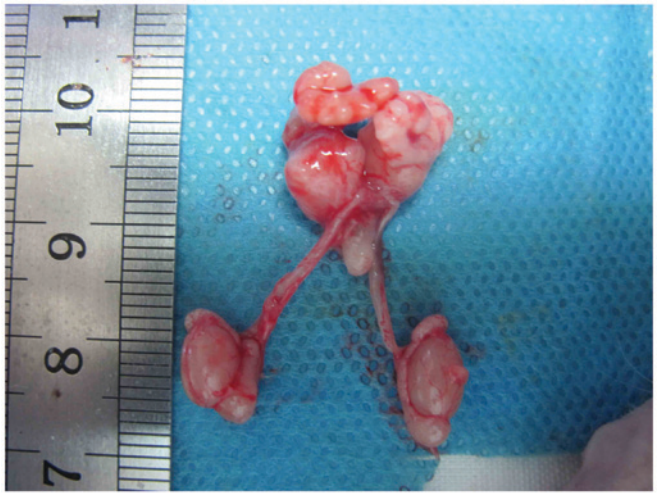

Figure 1. Orthotopic prostate cancer in a BALB/c nude mouse and the prostate tumor, which was excised 8 weeks following implantation from the ADPC group.

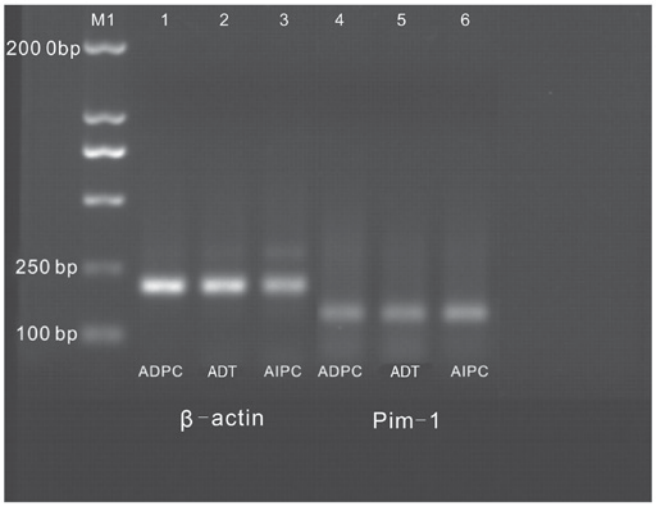

Figure 2. Agarose gel electrophoretogram demonstrating the relative mRNA expression level of Pim-1 in the ADPC, AIPC and ADT groups. ADPC, androgen-dependent prostate cancer; AIPC, androgen independent prostate cancer; ADT, androgen deprivation therapy.

secondary antibodies (dilution, 1:20,000; cat no. ab136636; Abcam) for 30 min at room temperature. After the application of the diaminobenzidine kit (dilution, 1:1; cat. no. SP-9000-D; Beijing Zhongshan Jinqiao Biotech Co., Ltd., Beijing, China), tissue sections were stained with hematoxylin, dehydrated and mounted. The slides were scanned with a Hamamatsu NanoZoomer scanner (Nikon ECLIPSE90ir; Nikon Corporation, Tokyo, Japan). Positive cells were defined as cell cytoplasm or nuclei that were immunostained dark yellow or brown. Microscopic imaging was used to quantify the positive immunoreactivity, which was recorded by a microscope equipped with a digital camera. Integrated optical density (IOD) was calculated to analyze semiquantitative expression of AR and Pim-1 using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). The immunoreactivity reaction and staining intensity of the prostate tumor cells were compared by calculating the mean optical density (MOD) from 15 different microscopic fields.

Statistical analysis. Statistical analyses were performed using SPSS software (version 14.0; SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference. The differences between multiple groups were determined by one-way analysis of variance followed by χ^2 analysis.

Results

RT-PCR electrophoretogram. The lengths of the amplified products of β-actin and Pim-1 were 205 and 147 bp, respectively. An agarose gel electrophoretogram revealed that the relative mRNA expression levels of Pim-1 in the ADPC, AIPC and ADT groups were 0.59±0.01, 1.14±0.015 and 0.62±0.026, respectively (Fig. 2). A statistically significant difference was observed for the ADPC and AIPC groups, as compared with the ADT group ($P<0.05$).

Amplification of RT-PCR. Only one Pim-1 amplification peak appeared during the entire amplification process, without non-specific peaks, non-specific amplification products or primer dimers. All amplification curves showed as typical S-shaped curves. The amplicon of the target gene, *Pim-1*, showed a rapid increase from the 20-25th cycles and the amplicon for the internal control gene, β-actin, appeared in

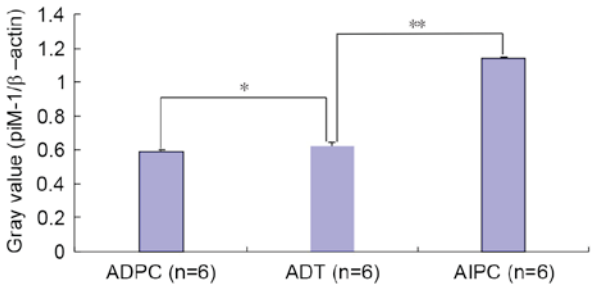

Figure 3. Expression level of Pim-1 mRNA was significantly different between the ADT, ADPC and AIPC groups. Data are presented as the mean ± standard deviation. $^{*}P<0.05$ and $^{**}P<0.01$. ADPC, androgen-dependent prostate cancer; ADT, androgen deprivation therapy; AIPC, androgen independent prostate cancer.

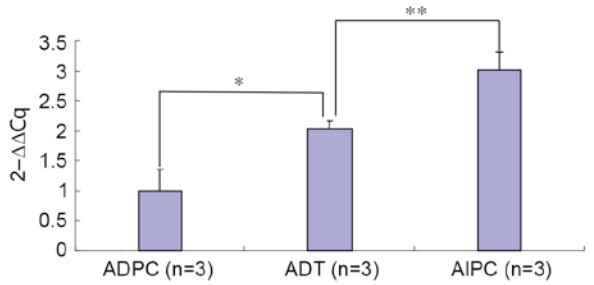

Figure 4. A statistically significant difference was identified among the ADPC, AIPC and ADT groups (n=3) by analysis of the Pim-1 amplification product using the $2^{-\Delta\Delta Cq}$ method. Data are presented as the mean ± standard deviation. $^{*}P<0.05$ and $^{**}P<0.01$. ADPC, androgen-dependent prostate cancer; AIPC, androgen independent prostate cancer; ADT, androgen deprivation therapy.

Table I. Cq value analysis of Pim-1 in the three groups.

Group	Pim-1 Cq value	β-actin Cq value	ΔCq value	ΔΔCq value	$2^{-\Delta\Delta Cq}$
ADPC	22.76±0.11	16.61±0.27	6.15±0.34[a]	0.00±0.10	1.00
ADT	22.69±0.38	17.58±0.20	5.11±0.21	-1.04±0.12	2.05
AIPC	22.51±0.45	17.95±0.46	4.56±0.23[a]	-1.59±0.30	3.01

Data are presented as the mean ± standard deviation. [a]P<0.05 vs. ADT. ADPC, androgen-dependent prostate cancer group; ADT, androgen deprivation therapy group; AIPC, androgen independent prostate cancer group.

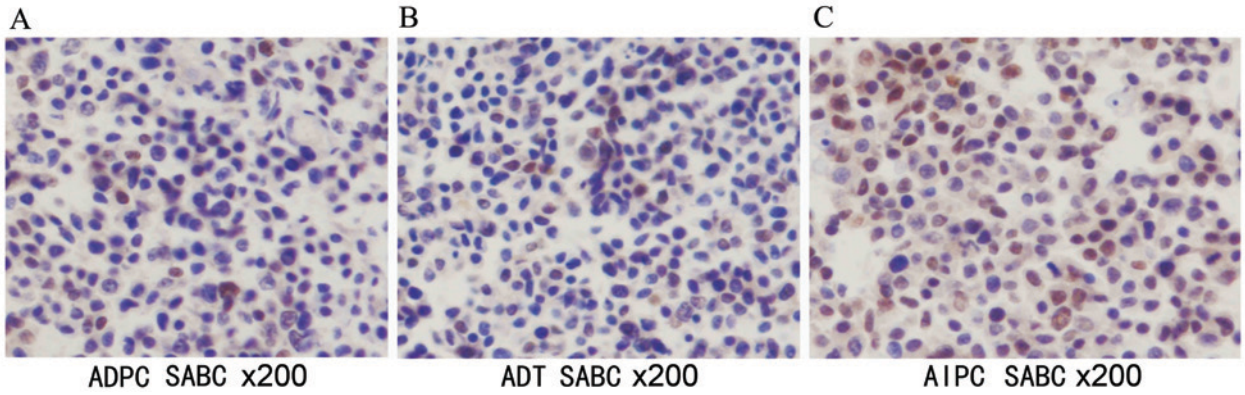

Figure 5. Immunohistochemical staining revealed the differences in AR expression in orthotopic prostatic implantation tumors from the three groups (magnification, x200). (A) Positive AR expression was observed in the nucleus of tumor cells in the ADPC group. (B) Positive AR expression was reduced markedly after ADT. (C) Positive AR expression was significantly increased in the AIPC group compared with the ADPC and ADT groups. ADPC, androgen-dependent prostate cancer; AIPC, androgen independent prostate cancer; ADT, androgen deprivation therapy; AR, androgen receptor; SABC, streptavidin-biotin complex.

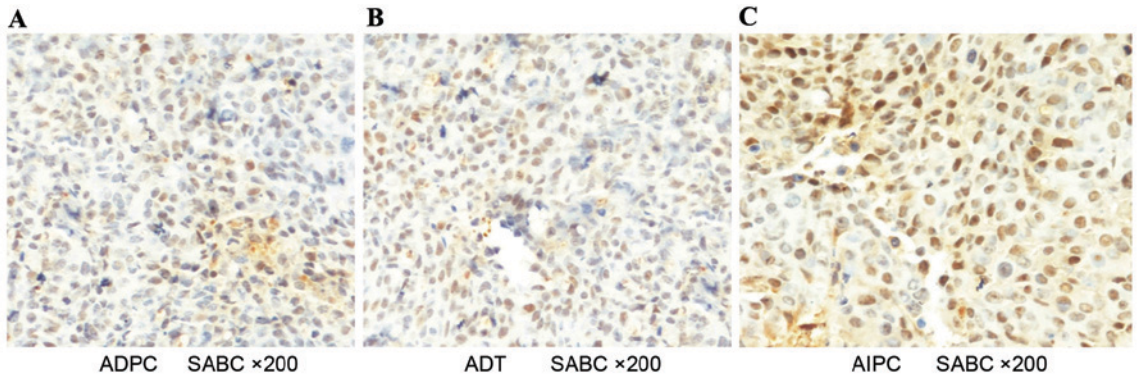

Figure 6. Immunohistochemical staining revealed Pim-1 expression was increased from ADPC to AIPC in orthotopic prostatic implantation tumors (magnification, x200). (A) Pim-1 was positive in the cytoplasm of tumor cells in the ADPC group. (B) Positive Pim-1 expression was increased following ADT. (C) The majority of the cancer cell cytoplasms were strongly stained for Pim-1 in the AIPC group. ADPC, androgen-dependent prostate cancer; AIPC, androgen independent prostate cancer; ADT, androgen deprivation therapy; SABC, streptavidin-biotin complex.

the 15-20th cycles. The expression level of Pim-1 mRNA was significantly different between the ADT and ADPC groups (P<0.05), and that of AIPC was higher than the ADPC group (Fig. 3; P<0.01). The ΔCq and ΔΔCq values of the *Pim-1* gene in the ADPC, ADT and AIPC groups are shown in Table I. Compared with the ΔCq value of Pim-1 in the ADT group, a significant difference was found in the ADPC group and AIPC group (P<0.05). Analyzed using the relative quantification method, the Pim-1 amplification product was increased by 2.05 and 3.01 times in the ADT and AIPC groups, respectively, as compared with the ADPC group (Fig. 4).

PSA concentration of mouse blood serum. The PSA concentration of the blank control group mice was 0 ng/ml; by contrast, that of the ADPC, ADT and AIPC groups were 0.48±0.025, 0.17±0.032 and 0.87±0.023 μg/l, respectively. The concentrations of the ADT and AIPC groups were significantly reduced and increased, respectively, as compared with the ADPC group (P<0.01).

Results of IHC. Histologically, AR was predominantly present in the cell nucleus (Fig. 5) and Pim-1 was present in the cytoplasm (Fig. 6). Image-Pro Plus software was used to analyze

Table II. Immunohistochemical-stained MOD of Pim-1 and AR expression in the three groups.

		MOD	
Group	n	Pim-1	AR
ADPC	15	0.017±0.0021[a]	0.032±0.009[a]
ADT	15	0.018±0.0013	0.019±0.006
AIPC	15	0.024±0.0019[a]	0.040±0.011[a]

Data are presented as the mean ± standard deviation. [a]P<0.05 vs. ADT. MOD, mean optical density; AR, androgen receptor; ADPC, androgen-dependent prostate cancer group; ADT, androgen deprivation therapy group; AIPC, androgen independent prostate cancer group.

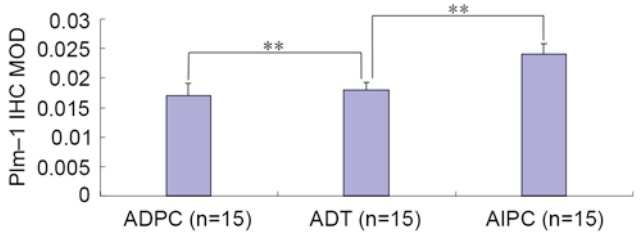

Figure 7. A statistically significant difference was identified among the ADPC, AIPC and ADT groups with regard to the MOD ratio of Pim-1 IHC staining results from 15 different microscopic fields. Data are presented as the mean ± standard deviation. **P<0.01. ADPC, androgen-dependent prostate cancer group; ADT, androgen deprivation therapy; AIPC, androgen independent prostate cancer; MOD, mean optical density; IHC, immunohistochemical.

the IHC results (Table II); a statistically significant difference was observed or the ADPC and AIPC groups, as compared with the ADT group with regard to the MOD ratio (Fig. 7).

Discussion

Prostate cancer remains the most common type of cancer in males in the USA and the western world. It was estimated that in the USA in 2016, prostate cancer would account for 21% of all newly diagnosed cancer cases representing 841,390 men. In 2016, 26,120 men in the USA succumbed to the disease (6). Prostate cancer mortality rates have been declining, which was attributed to PSA screening and androgen ablation treatment. Although most prostate cancer grow this dependent on the presence of androgens and initially responds well to ADT, the majority of cases progress to CRPC, which is currently incurable. An improved understanding of the underlying mechanisms of castration resistance is critical to reduce associated morbidity and mortality rates. There have been numerous studies attempting to clarify the mechanisms for the development of CRPC, including ligand-independent AR activation (7,8), AR mutations (9) or abnormal amplification of AR (10).

Pim is an oncogene that is closely associated with the proliferation and differentiation of cancer cells (11). They are primarily regulated at the transcription level without a specifically regulatory domain. *Pim* controls cellular proliferation, differentiation or apoptosis functions. Additionally, it regulates tumorigenesis via various signaling pathways. *Pim-1* is regulated by cytokines, growth factors or hormones, and it encodes a serine/threonine kinase, which is implicated in tumor malignant transformation and progression (12). For example, the overexpression of Pim-1 and Pim-2 are implicated in prostate cancer progression (13). To date, three members of oncogenic serine/threonine kinases genes, including the highly homologous Pim-1, Pim-2 and Pim-3, have been reported in the PIM family, and possess overlapping structures and functions. Among them, *Pim-1* has been widely investigated and its oncogenic nature has been confirmed (14). The crystal structure reveals that Pim-1 is a constitutively active kinase. Notably, it was reported that Pim-1 is predominantly located in the cytoplasm and nucleus, although it may also be found at the membrane. The *Pim-1* gene is a proto-oncogene that encodes two ubiquitous protein kinase isoforms, including Pim-1L (44 kDa) and PIM-1S (33 kDa), with high degrees of sequence and structural similarities. In humans, *Pim-1* is often expressed in normal and transformed cells, and its overexpression has been documented in various tumors (15). The expression level of *Pim-1* is higher in prostate cancer than in human benign prostatic hypertrophy (16). *In vitro*, the overexpression of exogenous Pim-1 increases prostate cancer cell proliferation and progression (17). *In vivo*, it has the potential to serve as a diagnostic and prognostic biomarker (18). According to clinical findings, *Pim-1* expression is associated with a poor prognosis and hormone insensitivity to ADT (11).

There are two Pim-1 kinase isoforms, namely Pim-1S and Pim-1L, which modulate AR stability and transcriptional activity (19); these isoforms modulate AR activity by phosphorylation of AR to promote prostate cancer cell growth (20). In a previous study, the decreased AR expression coincided with increased *Pim-1* expression, indicating that Pim-1 may contribute to regulation of AR turnover (21). AR is critical in prostate cancer development to CRPC, therefore, Pim-1 may serve as a potential target for the treatment of hormone-refractory prostate cancer (22). However, the molecular mechanisms of Pim kinases in specific signaling pathways for prostate cancer cell proliferation and progression are complicated and not well established (11). Pim kinases may be associated with the regulation of gene transcription through interactions with c-Myc and AKT to enhance tumorigenesis and inactivate cell cycle inhibitors by phosphorylating and downregulating p27Kip1 at the transcriptional and post-transcriptional levels (23,24). Furthermore, Pim may be involved in the regulation of cell proliferation and viability (14).

Due to the above-mentioned findings, Pim-1 is considered to be a potential tumor target for prostate cancer therapy. The development of specific Pim inhibitors is imperative for the treatment of CRPC patients. Notably, various Pim family small-molecule inhibitors targeting these kinases have been identified, and the majority of Pim kinase inhibitors are specific for Pim-1. These inhibitors display anticancer activity in prostate cancer cell lines, including those that are sensitive and resistant to chemotherapy (11). Despite the fact that the Pim-1 kinase inhibitors that have been investigated exhibit potential anticancer effects, there are a few small molecule inhibitors showing an inhibitory effect (25). In addition, other studies revealed that a Pim-1 specific monoclonal antibody markedly

inhibited the growth of the human prostate cancer cell line, DU145 in a mouse model and thus may be considered another potential treatment modality for CRPC (26). Furthermore, Pim-1 contributes to the regulation of DNA repair when CRPC is treated with paclitaxel. Cytotoxic drugs such as docetaxel activate Pim-1 kinase in DU145 cells (27). The ability of DNA repair significantly decreases in the absence of Pim-1, leading to severe DNA damage and apoptosis (22).

In the current study, subcutaneous planting and *in situ* embedding tumor methods were used to generate ADPC mouse models. Subsequently, surgical castration was performed to simulate ADT of human prostate cancer. PSA, AR and Pim-1 expression levels were analyzed using RT-qPCR, ELISA and IHC in three subgroups. The expression of AR and PSA revealed varying degrees of reduction in the ADT treatment subgroup; by contrast, the expression levels of AR and PSA were increased significantly in the AIPC subgroup, which was similar to the trend of ADT in human prostate cancer. These findings demonstrated that the *in situ* prostate cancer mouse model may successfully simulate an ADT model of humans. In the present mouse model, the expression levels of genes and proteins were significantly different in the ADPC and AIPC groups ($P<0.05$); Pim-1 was highly expressed and implicated in the AIPC model during ADT, which implied that the Pim-1 level was not influenced during ADT. It appeared that Pim-1 exerted a regulatory role in cell proliferation and tumor differentiation. Additionally, Pim-1may affect the ADT effect on the treatment of prostate cancer. The exact interaction mechanism of Pim-1 and AR remains unclear; the current findings revealed that the expression level of AR was decreased, while PIM-1 expression was elevated in the ADT subgroup. Notably, AR and Pim-1 were highly expressed in the AIPC subgroup. These findings revealed that there were high expression levels of Pim-1 during the ADT treatment period, indicating that Pim-1 is important in the progression and metastasis of prostate cancer. This requires further confirmation by subsequent experiments, such as using shRNA interference or Pim-1 inhibitors to decrease the expression of Pim-1 and systematically evaluate the mechanism of Pim-1 interaction with AR during ADT.

There were several limitations of the current study. First, we had to adjust the sample size from 8 to 6 for calculating statistical differences due to the successful operation rate of orthotopic implantation and to keep the same number of samples in the four groups. However, a sample size of less than 5 may yield a difference in subgroup analyses; a similar small sample especially in the animal model was observed in another study (28). In addition, there was lack of a Pim-1 inhibitor to treat the mice in order to observe the change during the ADT period. Furthermore, there was lack of Pim-1 data regarding metastatic tumors and a lack of tumor growth curves.

In conclusion, the expression level of Pim-1 was high during ADT, which may contribute to the progression or metastasis of prostate cancer; therefore, further studies are required to investigate the specific underlying mechanism of Pim-1 interaction with AR and ADT.

Acknowledgements

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